

Osteoarthritis and Cartilage



Evaluation of the inflammatory response in experimentally induced synovitis in the horse: a comparison of recombinant equine interleukin 1 beta and lipopolysaccharide

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SUMMARY

Objective: To compare two transient models of synovitis-osteoarthritis (OA) in horses by characterizing biological changes in synovial fluid and joint tissue.

Method: Twelve skeletally mature mares were utilized in a block design. Synovitis was induced by an intra-articular injection of 100 ng recombinant equine interleukin 1 beta (reIL-1 β) or 0.5 ng lipopolysaccharide (LPS) into a middle carpal joint in 1 ml volumes. One ml of saline was injected into the contralateral control joint. Lameness evaluations were conducted through post-injection hour (PIH) 8 (at which time arthroscopic removal of synovium and articular biopsies was done), and at PIH 240. Arthrocentesis collection of synovial fluid occurred between PIH 0 and 48. An arthroscopic examination at PIH 8 included synovium and articular cartilage biopsies for gene expression analysis.

Results: Synovial fluid analysis indicated that single injections of reIL-1 β or LPS increased synovial white blood cell (WBC), neutrophil count, total protein, prostaglandin E₂ (PGE₂) concentrations and general matrix metalloproteinase (MMP) activity relative to control joints through PIH 8. Injections of either reIL-1 β or LPS increased mRNA expression for MMP-1 and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS)-4 in synovium and for MMP-1, ADAMTS-4, ADAMTS-5 in articular cartilage collected at PIH 8 compared to saline injections.

Conclusion: Injections of reIL-1 β into equine carpal joints resulted in a transient inflammatory response that was similar in severity to the LPS injection, causing increased expression of certain deleterious mediators in joint tissues at 8 h. Given that IL-1 β is a known critical mediator of traumatic arthritis and OA, this humane and temporary model may be useful in evaluating therapeutics that act against early stages of joint disease.

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Introduction

Synovitis is a key factor in the pathophysiology of osteoarthritis (OA) in humans¹ and horses². The development of OA associated with synovitis is likely due to persistent upregulation of mediators that contribute to articular cartilage degradation including matrix metalloproteinases (MMPs), a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) and prostaglandin E₂ (PGE₂)². The cytokine interleukin-1 β (IL-1 β), which is prevalent in equine^{2,3} and human^{4–6} OA, stimulates production of MMPs and PGE₂⁴. IL-1 β 's importance in the OA cascade has been demonstrated by its

inhibition significantly reducing OA in well-established equine osteochondral fragment³ and canine anterior cruciate ligament⁷ models.

There are several similarities between horses and humans when considering OA. Both experience naturally occurring OA, due primarily to traumatic injury. The ability of inflammation to produce early signs of OA was demonstrated in horses over 30 years ago⁸. Given the importance of synovitis in the pathogenesis of OA, equine experimental models for inducing joint inflammation, including amphotericin, carrageenan, polyvinyl alcohol foam particles and blood, have been evaluated. The endotoxin lipopolysaccharide (LPS) from *Escherichia coli* bacteria is known to induce acute synovitis resembling septic arthritis, and has been used as a model^{9–14} to evaluate treatments for acute synovitis^{15–20}. The role of IL-1 β in the natural inflammatory cascade makes it a more appropriate model for evaluating the pathobiology of OA; however,

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IL-1 β use in *in vivo* experimental modeling has been minimal. Two studies have induced inflammation using intra-articular injections of recombinant human IL-1 β (rhIL-1 β)^{21,22}, reporting increased synovial white blood cells (WBCs) and total protein levels. Also, gene expression changes in rat joints following intra-articular injection of rat IL-1 β have been reported²³. While these studies suggest intra-articular injection of IL-1 β is suitable for evaluating OA pathobiology, a comprehensive profile of changes in both synovial fluid and articular tissue has not been done. Additionally, the effect of recombinant equine IL-1 β (reIL-1 β) has not been evaluated *in vivo* despite *in vitro* studies demonstrating a potent degradative response in articular cartilage explants at concentrations as low as 0.1 ng/ml²⁴. Therefore, we hypothesized that a single injection of reIL-1 β causes, (1) inflammatory changes in equine synovial fluid that are similar to LPS, and (2) gene expression changes in joint tissue that closely mimic early clinical OA.

Methods

Experimental design and induction of temporary carpal synovitis

Twelve healthy mares with clinically normal carpal joints, determined by radiographic evaluation, were blocked by age (mean = 9.8 years old), weight (mean = 540 kg) and body condition score²⁵ (mean = 7), and randomly assigned into LPS ($n = 6$) or reIL-1 β ($n = 6$) treatment groups. With the exception of the first author, all investigators and staff were blinded to the study.

Intra-articular injections were administered to the middle carpal joint in 1 ml volumes. At post-injection hour (PIH) 0, the LPS group received 0.5 ng LPS (from *E. coli* 055:B5; Sigma–Aldrich) in sterile phosphate buffered saline (PBS). The reIL-1 β group received 100 ng of reIL-1 β (R&D Systems) in sterile PBS. The reIL-1 β dose has been previously used in *in vivo*²⁶ and *in vitro*²⁴ models to elicit measurable inflammatory responses. As a control, each horse received an injection of sterile PBS into the contra-lateral carpal joint.

Evaluation of clinical response to treatment

Lameness measurements were conducted at PIH 0, 4, 8 and 240 using the American Association of Equine Practitioners grading scale²⁷. Horses were monitored throughout the study for signs of discomfort. Temperature, pulse, and respiration at rest were measured every 2 h through PIH 8; every 4 h through PIH 16, and at PIH 24 and PIH 48.

Synovial fluid collection

Synovial fluid was aseptically aspirated at PIH 0, 4, 8, 24 and 48. A portion of the fluid was processed for cytology (WBC count, cell differential and total protein). The remaining volume was centrifuged, and the supernatant was stored at -80°C until analyzed.

Arthroscopic biopsy procedure

At PIH 8, all horses underwent arthroscopic surgery on treated and control joints to obtain synovium and articular cartilage samples. Approximately 6 mg wet weight samples of both tissues were placed in 500 μl of TRIzol reagent (Invitrogen, Carlsbad, CA), flash frozen in liquid nitrogen and then stored at -80°C until analyzed.

Synovial fluid analysis

Synovial fluid WBC and total protein concentrations were determined using a Coulter T6–60 automated cell counter and

refractometry. Differential neutrophil values were evaluated using cytospin and direct smear analysis.

Synovial fluid samples were evaluated for PGE₂, general MMP activity and glycosaminoglycan (GAG) concentrations. Samples were analyzed in triplicate.

Synovial fluid PGE₂ was isolated by solid-phase extraction using C2 ethyl mini-columns (Agilent Technologies; Wilmington, DE), followed by quantification using a commercially available Enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs; Ann Arbor, MI).

Total MMP activity was determined based on cleavage of substrate FS-6 (Calbiochem, San Diego, CA), which has been validated for equine synovial fluid¹³. Negative controls that inhibited MMP activity were created with 10 mM ethylenediaminetetraacetic acid (EDTA). An EDTA-free protease inhibitor (complete[™], Roche Diagnostics GmbH; Mannheim, Germany) was used as a positive control to eliminate non-MMP enzymatic activity. Samples were aliquoted into black microplates, and fluorescence was read at excitation_{max} 324 and emission_{max} 400, every 5 min for 20 min. General MMP activity was determined by linear slope of increasing fluorescence with time, and expressed as a rate of FS-6 conversion per second (RFU/sec).

Total GAG concentrations were measured spectrophotometrically using a modified 1,9-dimethylmethylene blue dye-binding assay used previously in our laboratory²⁸.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of joint tissues

Cartilage samples were thawed, added to TRIzol reagent, and then pulverized. Synovial membrane samples were homogenized on ice for 30 s and mixed with TRIzol. To isolate RNA, a chloroform extraction procedure, previously used for joint tissue²⁹, was performed. Nucleic acid concentrations were determined using the ND1000 spectrophotometer at 260/280 nm. Samples with 260/280 ratios less than 1.7 were further purified using the RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to manufacturer's instructions. RNA was reverse transcribed to cDNA using Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). Primer/probes were mixed with TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and cDNA, and semi-quantitative real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primer/probes for ADAMTS-4, ADAMTS-5, MMP-1, MMP-13, IL-1 β , tumor necrosis factor- α (TNF- α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1) were designed and validated at the authors' institution. Primer/probes for cyclooxygenase-2 (COX-2) were purchased from the University of California-Davis's Lucy Whitter Molecular Core Facility (Davis, CA). Samples were analyzed in duplicate with expression normalized to the housekeeping gene GAPDH. Expression of each gene was calibrated to the average expression in the LPS control joints and reported as fold change relative to LPS controls.

Statistical analysis

Sample size was determined using SAS 9.2[®] statistical software (SAS Institute, Inc., Carey, NC) and data from previous research that measured synovial fluid total protein from horses that received LPS¹⁰, IL-1 β ²² or saline²². A sample size of 6 per treatment group provided a statistical power of 0.90 ($\alpha = 0.05$) as calculated using one-way analysis of variance (ANOVA). WBC, neutrophil cell count, and PGE₂ data were log transformed to standardize variance. All clinical and synovial fluid data were compared using ANOVA for repeated measures. Between-subject effects were treatment and joint with time being the repeated measures effect. Significance level was set at $P \leq 0.05$; differences were further analyzed using

Table 1
Primers and probes used for semi-quantitative real-time PCR

Target gene	PCR primers	PCR probe
GAPDH	Forward-AAGTGGATATTGTGCCATCAAT Reverse-AACTTGCCATGGGTGGAATC	TGACCTCAACTACATGGTCTAGTTTCA
ADAMTS-4	Forward-TGTGATCGTGTCTTGGCTCC Reverse-TGTTTGCTGCAGCTAGAACCATC	AGTTTGACAAGTGCATTGGTGTGCGGT
ADAMTS-5	Forward-AAGGTGACTGATGGGACCGAATGT Reverse-TTTGAGCCAATGATGCCGTTACAG	AGGCCATACAGTAATCCGCTCTGCGT
MMP-1	Forward-ACTGCCAAATGGACTCAAGCTGC Reverse-TCCTTCACAGTGCTAGGAAAGCCG	CAGGATGTGCTCTACGGATACCCCAAGGAC
MMP-13	Forward-TGATGAACTTGGACAAGCAGTTCC Reverse-CCTTGGAGTGGTCGAGACCTAAG	AGGCTACAACCTGTTTCTTGTGCTGCACAC
TNF- α	Forward-AGTGACAAGCCTGTAGCCCATGTT Reverse-ATGAGGTACAGCCCATCCAATGGT	AATGGCGTGAAGCTGACAGACAACCA
IL-1 β	Forward-AGTCTTCACTGCTCAGTTTCTGA Reverse-TGCCGCTGCAGTAAGTCATC	CAGCCATGGCAGCAGTACCCGA

Fisher's least squares means test. The data are presented as mean \pm 95% confidence interval.

Results

Clinical response to intra-articular injection

All horses were free of lameness (Fig. 1) prior to injection. By PIH 4 lameness was higher in all treated limbs (reIL-1 β : 3.7 ± 0.39 ; LPS: 3.8 ± 0.39) compared to baseline measurements ($P < 0.001$), which continued through PIH 8. Injections of PBS in the contralateral control joints did not result in lameness. Lameness evaluations were not performed at PIH 24 and 48 due to surgery, but at those time points all horses were bearing weight on limbs and walking comfortably. All horses were clinically sound 240 h post-injection.

Synovial fluid analysis

The volume of aspirated synovial fluid was significantly higher ($P < 0.0001$) in treated joints (4.49 ± 0.46 ml) than in control joints (2.87 ± 0.46 ml). Baseline measurement of synovial fluid WBCs [Fig. 2(A)] were similar among all horses and within normal ranges³⁰. Neutrophil values [Fig. 2(B)] at baseline differed amongst

horses; therefore, data were adjusted with baseline values as covariates. At baseline, two horses had significantly higher neutrophil values in treated joints compared to the remaining treated joints; however, baseline values were less than 1% of values obtained following treatment at PIH 4. In control joints, WBC concentrations were significantly different between reIL-1 β ($12,800 \pm 21,447$ cells/ μ l) and LPS joints ($1,000 \pm 21,447$ cells/ μ l) at PIH 8 ($P = 0.001$); Injections of reIL-1 β or LPS resulted in a significant increase in WBC count at PIH 4 (reIL-1 β : $134,300 \pm 19,581$ cells/ μ l; LPS: $143,283 \pm 19,582$ cells/ μ l) and 8 (reIL-1 β : $170,700 \pm 37,581$ cells/ μ l; LPS: $234,833 \pm 19,582$ cells/ μ l), respectively when compared to PBS control joints ($P < 0.001$) at similar time points [Fig. 2(A)]. At PIH 24 and 48, WBC concentrations in treated joints were not significantly different between reIL-1 β and LPS. A rise in neutrophils occurred in all joints above baseline values, with treated joints having significantly higher values compared to control joints ($P < 0.01$) [Fig. 2(B)]. Total protein [Fig. 2(C)], within normal levels³¹ prior to IA injections, was elevated significantly over baseline values at PIH 4 (reIL-1 β : 5.11 ± 0.61 g/dl; LPS: 5.45 ± 0.61 g/dl), PIH 8 (reIL-1 β : 6.1 ± 0.61 g/dl; LPS: 6.32 ± 0.61 g/dl), PIH 24 (reIL-1 β : 5.96 ± 0.61 g/dl; LPS: 6.38 ± 0.61 g/dl) and PIH 48 (reIL-1 β : 4.96 ± 0.61 g/dl; LPS: 5.08 ± 0.61 g/dl) in all treated joints ($P < 0.05$). Synovial fluid total protein levels in control joints, regardless of treatment, increased over time with concentrations peaking at PIH 24 (reIL-1 β : 5.28 ± 0.61 g/dl; LPS: 5.66 ± 0.61 g/dl).

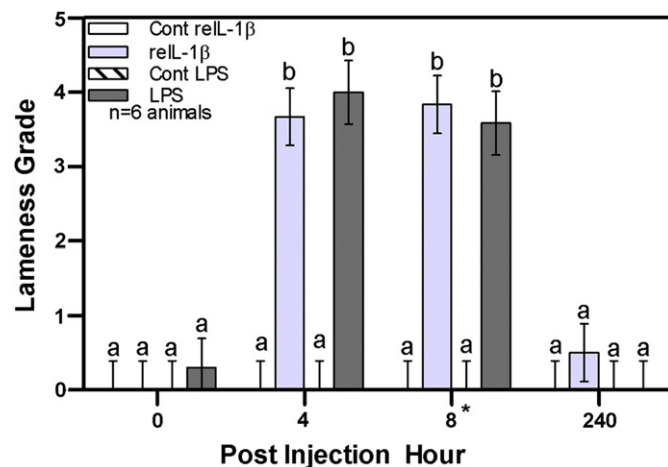


Fig. 1. Clinical response to intra-articular injection of reIL-1 β (100 ng), LPS (0.5 ng) or PBS on lameness grade. Columns lacking common letters indicate significant differences across both treatments (reIL-1 β , LPS) and time points ($P < 0.0001$). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means \pm 95% confidence intervals.

Synovial fluid biomarkers

Synovial PGE₂ concentrations [Fig. 3(A)] did not differ among horses at PIH 0. Injection of reIL-1 β or LPS increased PGE₂ concentrations (reIL-1 β : $1,952 \pm 357$ pg/ml; LPS: $2,244 \pm 450$ pg/ml) over control joints (reIL-1 β : 37 ± 357 pg/ml; LPS: 33 ± 330 pg/ml) by PIH 4 ($P < 0.01$). PGE₂ levels remained elevated through PIH 48 in all treated joints. Joints treated with reIL-1 β had significantly higher PGE₂ levels than LPS-treated joints at PIH 24 ($P = 0.011$) (reIL-1 β : 748 ± 329 pg/ml; LPS: 316 ± 329 pg/ml) and PIH 48 (reIL-1 β : 220 ± 329 pg/ml; LPS: 73 ± 329 pg/ml), respectively ($P = 0.001$).

Synovial MMP activity [Fig. 3(B)] was similar among all horses at PIH 0. Repeated fluid aspiration did not significantly change MMP activity in control joints. Synovial fluid from both treated groups demonstrated significantly higher MMP activity (reIL-1 β : 0.37 ± 0.13 Δ RFU/s; LPS: 0.44 ± 0.13 Δ RFU/s) compared to control joints at PIH 4 (reIL-1 β : 0.06 ± 0.14 Δ RFU/s; LPS: 0.06 ± 0.13 Δ RFU/s) ($P < 0.0001$). Levels were also significantly higher in treated joints (reIL-1 β : 0.61 ± 0.13 Δ RFU/s; LPS: 0.53 ± 0.13 Δ RFU/s)

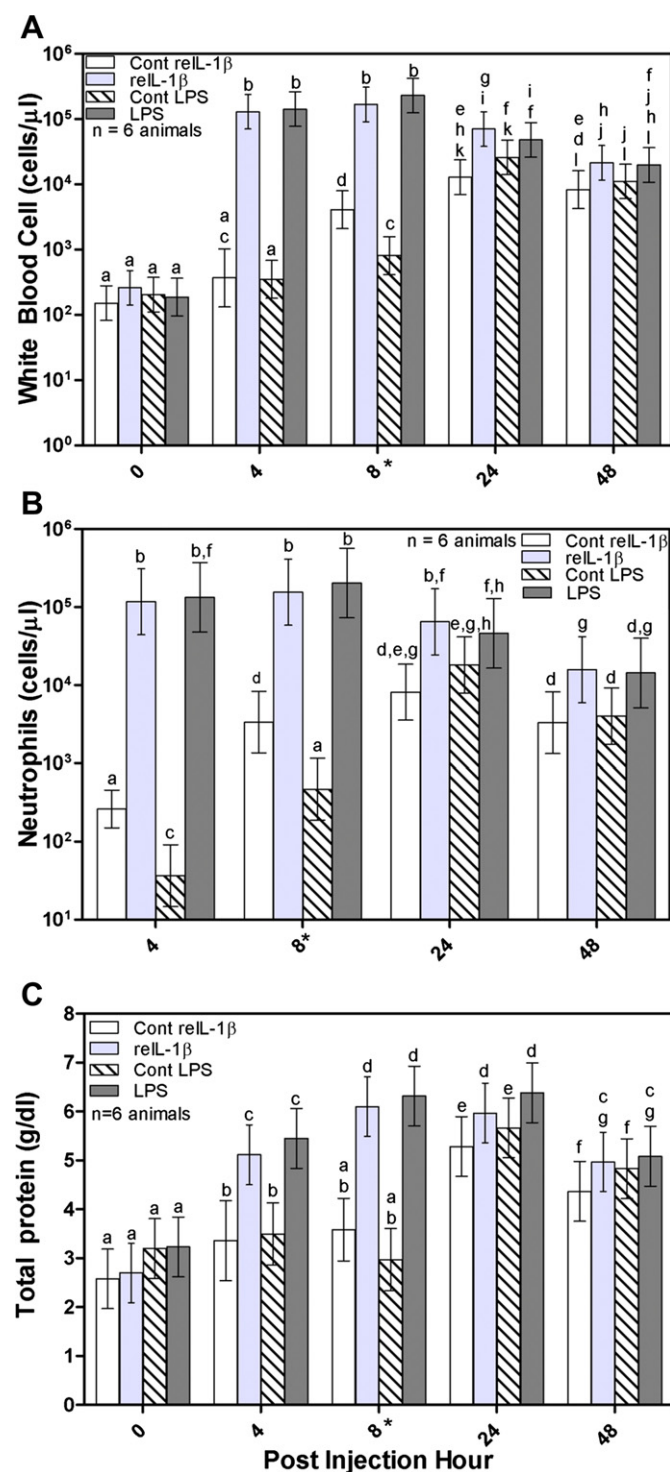


Fig. 2. Cytology evaluation of synovial response to intra-articular injection of reIL-1 β (100 ng), LPS (0.5 ng) or PBS. For each outcome, columns lacking common letters indicate significant differences across treatments (reIL-1 β , LPS), joints (treated, control), and time points. (A) WBCs ($P < 0.01$). (B) Neutrophils ($P < 0.01$). Neutrophil values at baseline differed among groups; therefore data were adjusted with PIH 0 values as covariates. (C) Total protein ($P < 0.0001$). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means \pm 95% confidence intervals.

compared to control joints (reIL-1 β : $0.07 \pm 0.13 \Delta$ RFU/s; LPS: $0.11 \pm 0.13 \Delta$ RFU/s) at 8 h post-injection ($P < 0.0001$). There were no significant differences between reIL-1 β and LPS groups at any time point.

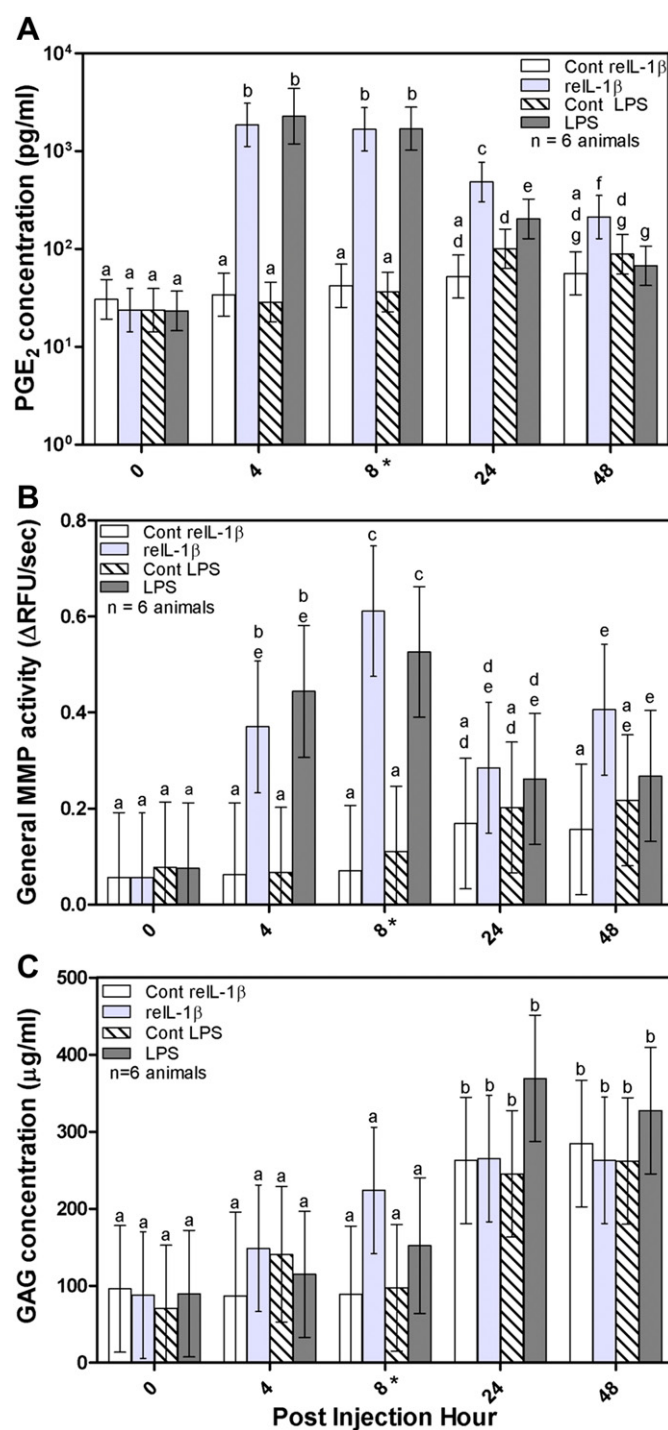


Fig. 3. Synovial fluid response to intra-articular injection of reIL-1 β (100 ng), LPS (0.5 ng) or PBS. (A) PGE₂ concentration: columns lacking common letters indicate significant differences across treatments (reIL-1 β , LPS), joints (treated, control), and time points ($P < 0.01$). (B) General MMP activity: columns lacking common letters indicate significant differences across joints (treated, control), and time points ($P < 0.0001$). (C) Glycosaminoglycan concentration: columns lacking common letters indicate significant differences across time points ($P < 0.0001$); significant difference over time across joints ($P = 0.051$). *Indicates time point when arthroscopic biopsy was performed. Data are displayed as means \pm 95% confidence intervals.

Synovial fluid GAG levels [Fig. 3(C)] were similar in all joints prior to injections, and remained similar through PIH 8. However, GAG levels, regardless of joint or treatment, increased at PIH 24, and remained elevated through PIH 48 ($P < 0.001$).

Gene expression in joint tissues at PIH 8

Synovium

Synovial tissue gene expression of MMP-13, ADAMTS-5, TNF- α , and COX-2 did not differ significantly among treated and control joints. There was a significant difference between LPS and reIL-1 β tissues compared to controls for MMP-1 (59-fold increase) ($P = 0.011$) and ADAMTS-4 (13-fold increase) ($P < 0.0001$) [Fig. 4(A and B)]. Expression of synovium IL-1 β mRNA [Fig. 4(C)] in treated joints was 5.5-fold higher than control tissues ($P = 0.003$) with LPS-treated joints having a 2-fold higher expression than reIL-1 β ($P = 0.038$).

Articular cartilage

Gene expression of MMP-13 and TNF- α was not significantly different among treated and control joints ($P = 0.18$). There was a significant difference between LPS and reIL-1 β tissues compared to controls for MMP-1 (223-fold increase) ($P = 0.002$) and ADAMTS-5 (3-fold increase) ($P = 0.002$) [Fig. 5(A and C)]. Expression of ADAMTS-4 [Fig. 5(B)] was 2.9-fold higher in LPS-treated cartilage vs reIL-1 β ($P = 0.054$) with treated joints 20-fold higher compared to control joints ($P < 0.0001$). Expressions of IL-1 β and COX-2 in control cartilages were not consistently within the detectable limit of the assay; therefore, these data were not analyzed. In cartilage from LPS joints, expression of IL-1 β and COX-2 were 3.4- and 3.9-fold higher in cartilage from reIL-1 β joints, respectively.

Discussion

The development of reversible *in vivo* models of synovitis is valuable for studying OA pathophysiology, and testing therapeutic modalities. The horse is an excellent translational model for evaluating OA treatments³ with outcomes relevant to human OA. The use of the endotoxin LPS has been an effective model for inducing joint inflammation^{11,12,16} and has been the primary agent used for *in vivo*, non-terminal equine studies. Comparative studies with other models are lacking, and little information is available regarding reIL-1 β 's ability to induce predictable inflammation in the joint. The reIL-1 β product has only recently become available

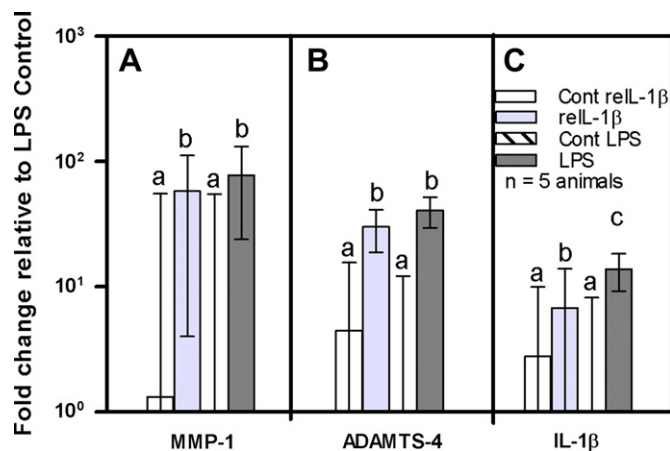


Fig. 4. Effect of single intra-articular injection of reIL-1 β (100 ng), LPS (0.5 ng) or PBS on synovium gene expression at 8 h post-injection. Values expressed as fold difference relative to LPS controls. (A) MMP-1 expression: columns lacking common letters indicate a significant difference between joints ($P = 0.011$). (B) ADAMTS-4 expression: columns lacking common letters indicate a significant difference between joints ($P < 0.0001$). (C) IL-1 β gene expression: columns lacking common letters indicate significant difference between joints ($P = 0.003$) and treatments ($P = 0.038$). Data are displayed as means \pm 95% confidence intervals.

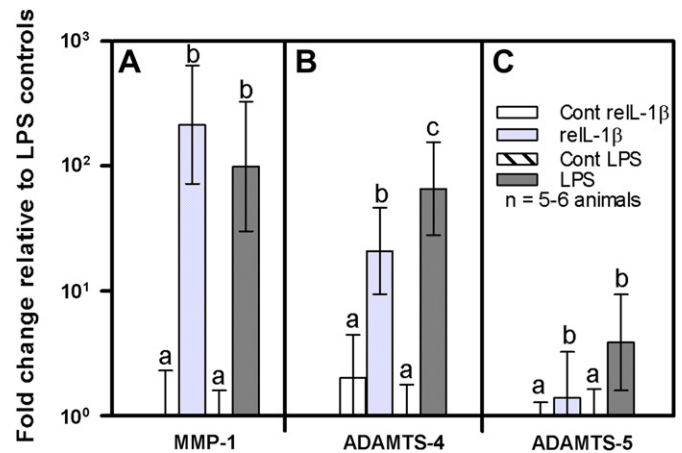


Fig. 5. Effect of single intra-articular injection of reIL-1 β (100 ng), LPS (0.5 ng) or PBS (controls) on cartilage gene expression at 8 h post-injection. Values expressed as fold difference relative to LPS controls. (A) MMP-1: columns lacking common letters indicate a significant difference between joints ($P = 0.002$). (B) ADAMTS-4: columns lacking common letters indicate significant difference between joints ($P < 0.0001$) and treatments ($P = 0.054$). (C) ADAMTS-5: columns lacking common letters indicate significant difference between joints ($P = 0.002$). Data are displayed as means \pm 95% confidence intervals.

and the *in vivo* use of intra-articular reIL-1 β has only been reported once²⁶ with results limited to defining inflammatory effects. Therefore, this study was aimed at comparing reIL-1 β to LPS as an *in vivo* synovitis model and exploring the potential of reIL-1 β as a reversible model of OA.

The AAEP lameness scoring system²⁷ is a clinical standard for classifying degrees of equine musculoskeletal injury and pain. The absence of lameness in the PBS-injected joints illustrated that it was the intra-articular injection of LPS or reIL-1 β that induced lameness. The lameness in horses treated with LPS was consistent with other studies that utilized a single dose of 0.5 ng LPS^{11,13}. Furthermore, 100 ng reIL-1 β and 0.5 ng LPS resulted in a similar degree of lameness, indicating reIL-1 β is equally sufficient as LPS for inducing clinical signs of lameness.

A strong intra-articular inflammatory response in treated joints was demonstrated by significant increases in synovial WBC, neutrophil counts and total protein between PIH 4–48, which was consistent with previous studies using exogenous LPS^{11–14}, rhIL-1 β ^{21,22}. In control joints, a significant increase in WBC concentrations at PIH 8, neutrophil counts at PIH 4, and total protein at PIH 4 are considered to be reflective of repeated arthrocentesis, which is consistent with other studies^{12,21}. The response to PBS was much less profound than that to LPS and reIL-1 β . Notably, at PIH 8, 6 ml of synovial fluid was easily aspirated from joints injected with LPS or reIL-1 β , and it was difficult to get more than 1 ml from control joints.

Synovial PGE₂ concentrations were elevated from intra-articular injections of reIL-1 β or LPS but not PBS. These results are consistent with previous studies using LPS^{12,13} or rhIL-1 β ^{21,22}. In addition, PGE₂ levels remained elevated in treated joints through PIH 48 when compared to baseline values; these findings differ from others using similar concentrations of LPS¹³ or rhIL-1 β ²². Hawkins and others¹² reported a comparable prolonged elevation in PGE₂ (beyond 8 h post-injection) to what was reported here; however, the LPS dosage (1.5 μ g) was higher than in the present study. Although one could expect the sustained PGE₂ concentrations in our study to reflect a secondary response due to surgery at PIH 8, PGE₂ levels in reIL-1 β control joints did not change significantly from baseline post-surgery (PIH 24–48) with surgical

manipulation. We conclude that a single 1 ml, intra-articular injection of 100 ng of reIL-1 β is adequate in stimulating *in vivo* PGE₂ production to levels that reflect an inflammatory response.

MMP activity in synovial fluid followed a similar pattern as PGE₂; joints treated with reIL-1 β or LPS exhibited increasing levels to PIH 8, and decreasing by PIH 24. While an increase in MMP activity was observed at PIH 48 in reIL-1 β treated joints, this increase was not at the magnitude seen at PIH 4, nor was it significantly different from LPS-treated joints at the same point in time. Synovial fluid collection did not resume until 16 h after surgery; therefore, it is unknown if the difference in MMP activity in treated joints is a result of treatment, effect of surgery or lag in time between collections. This was the first time arthroscopic biopsy had been introduced to either model so this requires further evaluation. Repeated aspiration did not alter MMP activity as evident by lack of significant change in control joints throughout the experimental period. These findings were in contrast to van den Boom and others³² who reported subsequent arthrocenteses increased MMP activity in unstimulated joints within 12 h. However, in that study, half of the horses were subjected to daily exercise; horses used in the current study were sedentary. Despite multiple collections, an intra-articular injection of 1 ml of PBS and repeated aspiration did not cause a significant change in MMP activity in control joints. The data presented here are the first to demonstrate reIL-1 β 's ability to instigate general MMP activity in the live animal, indicating reIL-1 β potential as a method for investigating inflammatory processes associated with early stages of OA.

In the current study, the arthroscopic surgical procedure at PIH 8 may have influenced MMP activity and GAG concentrations measured post-surgery. An increase in GAG concentrations in control and treated joints is speculated to be a consequence of the biopsy procedure rather than repeated aspiration or treatment application. It is unknown whether the overall change in GAG concentration is a reflection of increased synthesis or increased degradation. This could be clarified with the evaluation of additional anabolic and catabolic markers.

Synovial membrane and articular cartilage biopsies showed that intra-articular injection of LPS or reIL-1 β upregulated gene expression of certain catabolic enzymes associated with OA. The increase in MMP-1 expression with LPS or reIL-1 β is consistent with *ex vivo* stimulation of chondrocytes³³. To our knowledge, this study is the first to report *in vivo* upregulation of MMP-1 expression in equine joint tissue.

Intra-articular injection of reIL-1 β or LPS induced greater expression of ADAMTS-4, but not ADAMTS-5, in synovial membrane compared to control joints. These findings are consistent with ADAMTS-4 and ADAMTS-5 expression in human OA synovium³⁴. Articular cartilage expression of ADAMTS-4 and ADAMTS-5 is consistent with other equine³⁵ and human³⁶ OA models, yet differs from murine OA models in which ADAMTS-5 was the primary aggrecanase in cartilage degradation³⁷. Discrepancies between human and murine models have been reported³⁶ presenting a challenge in comparing findings in those species. However, significant expression of ADAMTS-5 in cartilage, but not in synovial tissue in the present equine study is similar to what has been reported in human studies (as reviewed)³⁸ indicating potential similarities in expression of aggrecanases between these two species.

The increase in IL-1 β expression in LPS-treated synovium compared to reIL-1 β treated joints may indicate an LPS-stimulated endogenous production of IL-1 β . It is unclear if the lower expression of IL-1 β in reIL-1 β treated synovium is due to a lack of or minimal endogenous IL-1 β production at PIH 8 that follows reIL-1 β injection. Previous *in vitro* studies have reported LPS-induced IL-1 β

production in human OA synovial tissue and cartilage^{39,40}; however measurements were of protein concentrations and not gene expression. Exogenous IL-1 β is rapidly cleared from the joint and does not stimulate endogenous production of IL-1 β ²¹, possibly allowing for a more representative evaluation of mediator response to IL-1 β . Concentrations of IL-1 β in synovial fluid were not evaluated in the present study; therefore, it is unknown if and to what extent endogenous IL-1 β production may have occurred in treated joints.

Upregulation of chondrocyte expression of ADAMTS-4 in LPS joints compared to reIL-1 β joints may be due to endogenous cytokine production stimulated by LPS injection; however, synovial fluid biochemical analysis did not differ between LPS and reIL-1 β .

The inflammatory response to LPS is characterized by an initial release of the cytokines IL-1 and TNF- α ^{4–6,41}, and is followed by a release of PGE₂^{12,13,17} and MMPs¹³. The two forms of IL-1 found in the horse are IL-1 α and IL-1 β ⁴². Both have been cloned and evaluated for stimulatory effect *in vitro*, and act through the same receptor, but equine IL-1 β is more potent for stimulating PGE₂ than equine IL-1 α ²⁴. The present study is the first to demonstrate that an injection of exogenous reIL-1 β resulted in significant increases in PGE₂ and general MMP activity.

To our knowledge, we report the first *in vivo* investigation of equine joint tissues in response to LPS or reIL-1 β injections. The tissue amounts collected through biopsy procedures were sufficient for evaluating mRNA expression with no permanent clinical signs consistent with morbidity to the defects. It is to be noted that the horses were not euthanized, a practice often required in other animal models²³. These findings further validate the role of synovitis in the generation of MMPs and aggrecanases that are associated with OA, and that MMP-1 and ADAMTS-4 may be therapeutic targets.

A limitation to this reIL-1 β synovitis model is that the injection of reIL-1 β overrides potential upstream regulators of IL-1 β synthesis in the natural disease process. However, given that inhibitors of IL-1 β have shown a strong ability to modulate cartilage degradation in experimental OA models^{41,7}, we feel that this model has great promise for identifying therapeutics that can significantly impact the progression of OA. The absence of protein measurements for synovial IL-1 β restricts our interpretation of LPS-stimulated cytokine activity, although based on gene expression analysis it appears LPS could stimulate IL-1 β protein synthesis. The inflammatory response was similar between reIL-1 β and LPS, as expected. LPS activates IL-1 β which further stimulates eicosanoid production and MMP activity. Additionally, the reaction by the synovial membrane is non-specific². Synovitis is related to acute stimulus rather than a specific cause.

We administered 100 ng of reIL-1 β as we believed that this dose would induce a robust inflammatory response that was temporary and not severe enough to require rescue analgesic, and had been the reIL-1 β dose used in a previous *in vivo* study²⁶. While a reIL-1 β dose of 0.1 ng/ml was reported to stimulate GAG release from equine cartilage explants²⁴ *in vitro*, a 6-fold increase in PGE₂ release was reported in the same study for 10 ng/ml of reIL-1 β . Given that PGE₂ release is an important outcome measure for models of synovitis, our dose was intended to stimulate robust PGE₂ release based on an estimated joint volume of 10 ml, resulting in a reIL-1 β concentration of 10 ng/ml upon injection. We confirmed lameness scores consistent with a robust yet humane inflammatory response. Additional studies would be needed to determine if a reIL-1 β dose less than 100 ng/ml could stimulate an inflammatory response that can be detected by changes in fluid biomarkers and tissue gene expression.

In this study, we characterized changes in synovial fluid and tissue following intra-articular injection of reIL-1 β that were found

to be consistent with known pathogenesis of OA, and are similar to the cellular response of acute synovitis commonly reported in equine OA. While the degree of inflammation may be reflective of sepsis or human rheumatoid arthritis, the response was short and temporary. With regard to the use of the contra-lateral joint as a control, it is to be noted that there was no increase in MMP and PGE₂ levels in those joints but there was an increase in white cell and GAG levels. Clarifying whether these changes were due to a systemic effect or to injection of saline would require a separate group of horses to be used as controls. The methods introduced in this study produced a synovitis response in horses that were temporary and reflective of OA, supporting the use of the horse as a translational model for studying OA.

Contributions

All authors contributed to the research project's design and conception; data analysis and interpretation; and in the writing, revising and final approval of the manuscript.

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Conflict of interest

None of the authors have conflicts of interest related to the manuscript.

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Animal study compliance

The research protocol (IACUC#09-1523) was approved by the CSU Institutional Animal Care and Use Committee.

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